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TITLE:

Leukodepletion Filters-Derived CD34+ Cells as a Cell Source to Study Megakaryocyte Differentiation and Platelet Formation

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SUMMARY:

This protocol describes in detail all the steps involved in obtaining leukofilter-derived CD34+ hematopoietic progenitors and their *in vitro* differentiation and maturation into proplatelet-bearing megakaryocytes that are able to release platelets in the culture medium. This procedure is useful for in-depth analysis of cellular and molecular mechanisms controlling megakaryopoiesis.

ABSTRACT:

The *in vitro* expansion and differentiation of human hematopoietic progenitors into megakaryocytes capable of elongating proplatelets and releasing platelets allows an in-depth study of the mechanisms underlying platelet biogenesis. Available culture protocols are mostly based on hematopoietic progenitors derived from bone marrow or cord blood raising a number of ethical, technical, and economic concerns. If there are already available protocols for obtaining CD34 cells from peripheral blood, this manuscript proposes a straightforward and optimized protocol for obtaining CD34+ cells from leukodepletion filters readily available in blood centers. These cells are isolated from leukodepletion filters used in the preparation of blood transfusion products, corresponding to eight blood donations. These filters are meant to be discarded. A detailed procedure to collect hematopoietic progenitors identified as CD34+ cells from these filters is described. The method to obtain mature megakaryocytes extending proplatelets while discussing their phenotypic evolution is also detailed. Finally, the protocol present a calibrated pipetting method, to efficiently release platelets that are morphologically and functionally similar to native ones. This protocol can serve as a basis for evaluating pharmacological compounds

acting at various steps of the process to dissect the underlying mechanisms and approach the *in vivo* platelet yields.

INTRODUCTION:

Blood platelets come from specialized large polyploid cells, the megakaryocytes (MK), that originate from a constant and fine-tuned production process known as megakaryopoiesis (MKP). At the apex of this process are hematopoietic stem cells which, in contact with the bone marrow environment (cytokines, transcription factors, hematopoietic niche), will be able to proliferate and differentiate into hematopoietic progenitors (HP) able to commit toward the megakaryocytic pathway, giving rise to immature MKs[1]. Under the influence of various cytokines, and in particular thrombopoietin (TPO), which is the major cytokine of MKP; the MK will then undergo two major stages of maturation: endomitosis and the development of demarcation membranes (DMS). This fully mature MK then appears close to a sinusoid vessel in which it can emit cytoplasmic extensions, the proplatelets, which will be released under the blood flow and subsequently remodeled into functional platelets[2]. The cloning of TPO in 1994[3] provided a boost in the study of MKP by accelerating the development of *in vitro* culture techniques allowing HP differentiation and MK maturation.

There are many pathologies affecting blood platelets, both in terms of platelet number (increase or decrease) and function[4,5]. Being able to recapitulate MKP *in vitro* from human HP could improve understanding of the molecular and cellular mechanisms underlying this process and ultimately the therapeutic management of patients.

Various sources of human HP are suitable: cord blood, bone marrow, and peripheral blood[6–8]. Harvesting HP from peripheral blood raises less logistical and ethical problems than their recovery from cord blood or the bone marrow. HP can be recovered from leukapheresis or buffy coat, but these sources are expensive and not always available in blood centers. Other protocols, less expensive and easier to perform, allow direct recovery of human peripheral blood mononuclear cells (PBMCs) without the need for prior CD34 driven isolation[4,8]. However, the purity of megakaryocytes is not satisfactory with this method and a selection of CD34+ cells from PBMC is recommended for optimal differentiation into MK. This led us to implement a HP purification from leukoreduction filters (LRF), routinely used in blood banks to remove white blood cells and thus avoid adverse immunological reactions[9]. Indeed, since 1998, platelet concentrates have been automatically leukodepleted in France. At the end of this process, LRF are discarded and all the cells retained in the LRF are destroyed. Cells in LRFs are, therefore, readily available at no additional cost. LRFs have a cellular content close to that obtained by leukapheresis or in buffy coats, notably in their composition of CD34+ HP making them a remarkably attractive source[10]. LRF as a human HP source has already been demonstrated to provide cells with intact functional capacities[11]. This source has the advantage of being abundant and affordable for laboratory research. In this context, this article describes successively: i) the extraction and selection of $CD34^+$ HP from LRFs; ii) a two-phase optimized culture, which recapitulates the commitment of HP into the megakaryocytic pathway and the maturation of MK capable of emitting proplatelets; iii) a method for efficiently releasing platelets from these MK; and iv) a procedure for phenotyping MK and cultured platelets.

PROTOCOL:

Control human samples were obtained from volonteer blood donors who gave written informed consent recruited by the blood transfusion center where the research was performed (Etablissement Français du Sang-Grand Est). All procedures were registered and approved by the French Ministry of Higher Education and Research and registered under the number AC_2015_2371.The donors gave their approval in the CODHECO number AC- 2008 - 562 consent form, in order for the samples to be used for research purposes. Human studies were performed according to Helsinki declaration.

1. Extraction and selection of CD34+ cells (HP) from LRF

1.1.**Reagent's preparation** (for one LRF)

1.1.1. Prepare 25 mL of filtered elution buffer: 21.25 mL of Phosphate Buffered Saline (PBS), 2.5 mL of Acid-Citrate-Dextrose (ACD) and 1.25 mL of decomplemented Fetal Bovine Serum (FBS). Filter on 0.22 µm and place at 37 °C.

1.1.2. Prepare 500 mL of PBS with 2 mM of ethylenediaminetetraacetic-acid (EDTA).

1.1.3. Dispose 25 mL of density gradient medium (DGM) (1.077 g/mL) in two 50 mL tubes.

1.2. LRF back-flushing modalities (Figure 1)

NOTE: This step requires a sterile tube welding machine, allowing sterile connection of thermoplastic tubing.

1.2.1. First, connect the LRF to an empty 600 mL transfer bag and the LRF to a tubing set (**Figure 1A**). Under the biosafety cabinet, inject the total volume of filtered and prepared elution buffer corresponding to the number of LRFs handled (xLRF x 25 mL) into the empty bag. Then, using a 30 mL syringe to gently aspirate the entire contents of the bag through the LRF, backflush and transfer the cells into a new 50 mL tube (**Figure 1B**).

1.2.2. To the red blood cells sedimentation, dilute the cell suspension by half with Dextran 2% and mix well to aggregate red blood cells. Wait for 30 min at room temperature (RT).

[Place figure 1 here]

1.3. PBMC collection

1.3.1. Following the red blood cells sedimentation, remove and transfer the supernatant into a 50 mL tube and fill it with PBS-EDTA 2 mM. Gently overlay the supernatant onto the above-prepared DGM. Let the supernatant flow gently without breaking the surface plane of the density gradient. Centrifuge at 400 x *g* at RT for 30 min in brake off mode.

1.3.2. Collect the PBMC layer with a disposable transfer pipette. Transfer the cells from each DGM tube into a new sterile 50 mL tube. Fill each tube with PBS-EDTA 2 mM and wash twice in 50 mL of PBS-EDTA 2 mM at 200 x *g* for 10 min at RT in brake on mode.

1.3.3. Pipette off and pool the cell pellet with 50 mL PBS-EDTA 2 mM.

NOTE: There is a possibility to stop the procedure by maintaining the collected cells under agitation at 4 °C during the night. Then, filter the suspension with a 40 µm cell strainer to remove the aggregates formed.

1.4. CD34+ cells selection

1.4.1. Determine the cell number and centrifuge at 400 x *g* at room temperature for 10 min with the break on.

1.4.2. Aspirate the supernatant completely and resuspend in the appropriate volume of PBS-EDTA 2 mM detailed by the manufacturer of the CD34 selection kit (300 µL of PBS-EDTA for 10^8 cells). Add the FcR blocking Reagent and the CD34 Microbeads in the appropriate concentration (50 µL for 10^8 cells).

1.4.3. After 30 min at 4 °C, wash the cell suspension and resuspend in the appropriate volume of PBS-EDTA 2 mM detailed by the manufacturer of the CD34 selection kit (500 µL per 10^8 cells).

NOTE: The selection is made on sorting columns for the passage of maximum 2 x 10^9 cells per column.

1.4.4. Pass the sample over the wet column of the magnet. Wash twice with 3 mL of PBS-EDTA 2 mM and elute the cells with 5 mL of PBS-EDTA 2 mM. A second run on a new column following the same procedure is necessary to improve the purity of the sample.

NOTE: An expected number of 6.1 x 10^5 cells/LRF (**Figure 2A**). For higher LRF numbers, scale up reagents and methods accordingly.

1.5. Evaluation of CD34+ cell purity

1.5.1. Add to an aliquot of 100 µL of suspension obtained after the $CD34^+$ selection, 2 µL of human CD34-PE antibody or 2 µL of IgG – PE (control). Mix well and incubate for 15 min at 4 °C.

1.5.2. Wash cells by adding 2 mL of PBS and centrifuge at 400 x *g* for 5 min. Aspirate supernatant completely and resuspend in 200 µL of PBS.

1.5.3. Analyze the purity by flow cytometry as shown in **Figure 2A** and in the **Discussion**.

NOTE: A purity of CD34+ cells above 90% is expected (**Figure 2Bii**).

1.5.4. Use the CD34+ cells directly or freeze for further use.

1.6. CD34+ cells freezing

NOTE: CD34+ cells freezing is done at a density of 10^6 cells per mL.

1.6.1. Following the CD34+ cell number determination, prepare the following cryopreservation media: (1) 60% Stemspan + 40% FBS, (2) 40% Stempsan + 40% FBS + 20% Dimethyl Sulfoxide (DMSO) and allow cooling at 4 °C.

1.6.2. Centrifuge the CD34+ cells at 400 x *g* at room temperature for 5 min and resuspend the pellet in cold solution 1 and then immediately add to the cold solution 2 (v/v).

1.6.3. Place the cryotubes immediately into a -80 °C freezer for 24 h and then transfer cryotubes into the liquid nitrogen tank.

2. Culture and differentiation of CD34+ cells to produce mature proplatelet-bearing megakaryocytes

NOTE: Cell culture protocol (**Figure 3A**), representative scheme of the cell culture procedure are detailed in this section.

2.1. CD34+ cells thawing (if required)

2.1.1. Prepare the thawing solution: 13 mL of PBS-20% FBS and place at 37 °C for 15 min. Quickly transfer the cryotubes to a 37 °C water bath until only one small ice crystal. Under the biological culture cabinet, pipette the whole content, and slowly transfer in 13 mL of prewarmed thawing solution.

2.1.2. Determine the cell number and cell viability.

2.2 Culture protocol, step 1: from day 0 to day 7

NOTE: Usually cultures are made in 24-well plates with 1 mL of medium per well at a density of 40,000 viable cells/mL, corresponding to 20,000 viable cells/cm². It is crucial to respect this density if any scale up is planned.

2.2.1. Growth medium preparation**:** In serum-free hematopoietic cell expansion media (previously heated to 37 °C) add Penicillin-Streptomycin-Glutamine (PSG) 1x, human Low-density Lipoprotein (hLDL) at 20 µg/mL, cytokine cocktail of megakaryocyte expansion 1x and Stemregenin 1 (SR1) at 1 µM.

2.2.2. Cell seeding**:** Centrifuge the thawed CD34+ cells at 400 x *g* at room temperature for 5 min. Thoroughly remove the supernatant and resuspend the cell pellet in 1 mL of culture media and performa cell numeration and viability to determine the appropriate volume to seed the cells.

2.2.3. Centrifuge the cells 400 x *g* at room temperature for 5 min and resuspend the pellet in the appropriate volume of the warm growth medium. Incubate the cells at 37 °C with 5% CO_2 for 7 days.

2.3. **Culture protocol, step 2: from day 7 to day 13 (Figure 3B, representative images at day 13)**

NOTE: Usually cultures are made in 24-well plates with 1 mL of medium per well at a density of 50,000 viable cells/mL, corresponding to 25,000 viable cells/cm². It is crucial to respect this density if any scale up is planned.

2.3.1. Maturation medium preparation: In serum-free hematopoietic cell expansion media (previously heated to 37 °C) add PSG 1x, hLDL at 20 µg/mL, TPO at 50 ng/mL, and SR1 at 1 µM.

2.3.2. Examine the cells under the microscope. At day 7, cells display a round and homogeneous appearance by filling the wells or the flasks without being too confluent.

2.3.3. Under a biosafety cabinet, gently transfer the cells in a 15 mL tube. Wash wells with PBS. Then, determine the number of cells and their viability to calculate the appropriate volume to seed the cells.

2.3.4. Centrifuge the cells at 400 x *g* at room temperature for 5 min. Remove the supernatant and resuspend the cells in the appropriate volume of warm media calculated in the previous step. Incubate the cells at 37 °C, 5% CO_2 for 6 days.

2.4. Cultured platelet release at day 13

2.4.1. Add 0.5 µM of prostaglandine I_2 (PGI_2) and 0.02 U/mL of apyrase to the culture and perform successive pipetting five times with a 1 mL pipette.

NOTE: Platelets are now released into the medium.

3. Flow cytometry analysis (MK phenotyping and cultured platelets count)

NOTE: This protocol can be applied to the phenotyping of the cells on the selected culture days. It also allows the determination of the number of the cultured platelet release (**Figure 4A,B**).

3.1 Preparation for MK analysis

3.1.1. Label four sets of microcentrifuge tubes as follows: Unlabeled cells as control, cells + 5 µL CD41 – Alexa Fluor 488, Cells + 5 µL CD34 – PECy7, Cells + 5 µL CD41 – Alexa Fluor 488 + 5 µL

CD34 – PECy7. Use a minimum of 1.10^5 cells per tube, do not exceed 1.10^6 cells per tube. Add to 100 µL of cell suspension per cytometry tube and the different antibodies. Incubate in the dark for 30 min at 4 °C.

3.1.2. Then, add 2 mL of PBS-EDTA 2 mM per tube and centrifuge at 400 x *g* for 5 min at room temperature. During centrifugation, prepare a solution of PBS-EDTA 2 mM + 7-Aminoactinomycin-D (7AAD) (1/100), allow for 300 µL solution per tube.

3.1.3. Remove the supernatant and take up the pellet in 300 µL of PBS-EDTA 2 mM with 7AAD. Run samples through the flow cytometer within 30 min.

NOTE: Analysis strategy for flow cytometry is shown in the **Figure 3C** and in the **Discussion**.

3.2 Tube preparation for cultured platelet analysis

3.2.1. Label four sets of microcentrifuge tubes as follows: Unlabeled cells as control, Cells + 5 µL CD41 – Alexa Fluor 488, Cells + 20 µL CD42a – PE, Cells + 5 µL CD41 – Alexa Fluor 488 + 20 µL CD42a – PE. Following five successive pipetting in culture well, transfer 300 µL of the suspension in tube for cytometry containing a calibrated number of fluorescent beads.

3.3.2. Add antibodies and incubate in the dark at RT for 30 min.

3.2.3. Run the samples through the flow cytometer within 30 min and set the acquisition for the passage of 5,000 beads.

NOTE: Analysis strategy for flow cytometry is shown in the **Figure 4B** and in the **Discussion**.

REPRESENTATIVE RESULTS:

Extraction and selection of CD34+ cells from LRFs

Here, the method, derived from Peytour et al.[9], describes the extraction and selection of $CD34^+$ cells from discarded LRFs available in blood banks after leukocyte removal. Following the backflush procedure, usually 1.03×10^9 to 2.45×10^8 cells/LRF (Mean±SEM; n = 155) are recovered with a viability of 94.88 ± 0.10% (**Figure 2A i**). After the CD34 positive selection, an average of 615.54 ± 12.28 cells/LRF is obtained (n = 155) (**Figure 2A ii**). If the number of cells is less than 300,000, it must be concluded that the procedure has not been carried out correctly and must be stopped. To evaluate the success of the CD34 selection, the purity of CD34+ cells is assessed by flow cytometry (**Figure 2B**). Routinely, a purity above 90% (91.88 ± 0.79%) is expected (**Figure 2B**). A purity below 75% could mean that there has been a problem in conducting the protocol and in particular the elution of the columns. Below a 75% purity the cells are not conserved for culture experiments.

[Place figure 2 here]

Differenciation and maturation of MK-bearing proplatelets

The cell culture procedure described is divided in two steps. The first one, from day (D) 0 to D7, is dedicated to HP proliferation and commitment into the megakaryocytic pathway in response to a combination of cytokines and the addition of a chemical compound SR1. The second one, from D7 to D13, is focused on MK maturation and proplatelet extension following the addition of TPO and SR1 (**Figure 3A**). As quality control of the culture, cell counting, determination of cell viability, and phenotyping of cells, are perfomed at D7 and D10. These stages have been chosen because they are crucial for HP commitment, D7 and MK maturation, D10, respectively (personal data). At D7 and 10, proliferation is routinely of x4.16 ± 0.25 (n = 34) and x2.30 ± 0.16 (n = 5), respectively, with a cell viability comprised between 86.38 ± 0.73% (n = 34); and 84.80 ± 2.67% (n = 5) (**Figure 3B**). Concerning cell phenotyping, as shown in **Figure 3C**, at D0, more than 90% of the cells are positive for CD34. Then, CD34+ cells become committed toward the megakaryocytic lineage, as witnessed by the apparition of CD41, a specific and early marker of MKP. Indeed, at D7, 50.20 ± 2.90% of the cells are positive for both CD34 and CD41 (**Figure 3Bii**). Then, MK improves their maturation. At D10, a majority of MK are mature, with less than 15.60 ± 4.70% of the cells being negative for CD41, 47.90 ± 8.90% being $CD34^{+}CD41^{+}$ and 36.40 ± 12.40% being $CD34^{-}CD41^{+}$ (**Figure 3Biii**).

[Place figure 3 here]

Cultured platelets release, day 13

Examination of the wells at D13 shows round MK and proplatelet-bearing MK (**Figure 4A**). An average of 35% of cultured MK extend proplatelets[12]. Of note, D13 represents the optimal day for proplatelet extension and platelet release. If the required level of MK capable of emitting proplatelets is not reached, something must have gone wrong along the culture process and the results should not be taken into account.

Although the exact mechanisms promoting platelet release from mature MK are still poorly understood, it is well known that hemodynamic forces are indispensable. To mimic these forces *in vitro*, the suspension containing proplatelet-bearing MK is aspirated and repelled five times with a P1000 cone and then analyzed by flow cytometry. For this purpose, tubes containing a calibrated number of fluorescent beads are used. First, beads present into the tube are gated on the CD41-Alexa-fluor 488/PErcP-Cy5 window (**Figure 4Bi**, in red). Then, cultured platelets are visualized in a pre-gate (platelet-like elements), determined on the forward scatter (FSC) and side scatter (SSC) parameters of native platelets (**Figure 4Bii**). The number of platelets is then determined on their CD41/CD42a positivity (**Figure 4Biii**). Cell counting is stopped in this protocol at 5,000 beads but another fixed number may be used depending on the supplier's recommendation. From the data acquired, the number of platelets counted per 5,000 beads is routinely at an average of 24.01 ± 92 (n = 15) (**Figure 4C**). Knowing the volume aspirated by the flow cytometer to count 5,000 beads (to be calculated for each cytometer) and the total volume of culture, it is possible to obtain an approximation of the total number of cultured platelets released.

[Place figure 4 here]

The procedure at a glance

To better summarize the method and understand each step, a poster summarizing the protocol step by step is presented in **Figure 5**. This summary sheet can be displayed in the culture room and serve as a memo. Of note, the success of the experiments is only guaranteed with the product references indicated in the table provided.

[Place figure 5 here]

FIGURE AND TABLE LEGENDS:

Figure 1: LRF back flushing modalities. (**A**) Representative scheme of (**i**) the sterile connection of the transfer bag to the LRF and (**ii**) the tubing set to the LRF. (**B**) Representative scheme of the connection of the syringes for cell collection.

Figure 2: CD34+ cell number/LRF and CD34 purity analysis. (**A**) (**i**) An analysis, by cell counter, of the number of cells and their viability obtained following the procedure including both PBMC collection and CD34 selection is performed (($1.03.10^9 \pm 2.45.10^8$ cells/LRF (Mean ± SEM; n=155) with a viability of 94.88 ± 0.10% (n = 155)). (**ii**) An analysis, by cell counter, is also performed after CD34 selection (615,54 ± 12.28 cells/LRF (n = 155)). (**B**) CD34 Purity is analyzed by flow cytometry. (**i**) Cells were stained with a CD34-PE antibody and identified on their FSC/SSC parameters. (**ii**) Based on the scatter signal and CD34 expression analysis, the purity is determined. A pre-gate of CD34 positivity was used based on the negative control for the CD34 marker. (**iii**) As can be seen on the bar graph, a purity of CD34+ cells above 90% (91.88 ± 0.79% (n = 17)) is expected.

Figure 3: Differenciation and maturation of MK-bearing proplatelets. (**A**) Representative scheme of the cell culture procedure. A two-step method is used: a proliferation step from D0 to D7 (SR1 and cocktail of cytokines) and a maturation step from D7 to D13 (SR1 and TPO). At D13, cultured platelets can be released following five successive pipetting. (**B**) (**i**) Proliferation rate at D7 (x4.16 ± 0.25 (n = 34)) and cell viability (86.38 ± 0.73% (n = 34)). (**ii**) Proliferation rate at D10 (x2.30 ± 0.16 (n = 5)) and cell viability (84.80 ± 2.67% (n = 5)). (**C**) Flow cytometry analysis stategy of the phenotypic evolution of MK in culture. At D0, 95.80 ± 0.80% of the cells are CD34 positive (n = 3). At D7, 50.20 ± 2.90% of the cells are positive for CD34 and CD41. At D10, less than 15.60 ± 4.70% of the cells are negative for CD41.

Figure 4: Cultured platelets release at day 13. (**A**) Representative light microscopy image of MK emitting proplatelets at D13. (**B**) Strategy to quantify cultured platelet release. (**i**) Beads are gated on the CD41-Alexa-fluor 488/PErcP-Cy5 window (in red). (**ii**) Platelet-like elements are visualized in a gate determined on the FSC/SSC parameters of native platelets (grey dots). (**iii**) Cultured platelets are determined on their CD41/CD42 positivity (purple). (**C**) Number of platelets counted per 5,000 beads can be obtained, routinely an average of 24,011 ± 919 (n = 15).

Figure 5: Isolation of $CD34^+$. Poster summarizing the protocol step by step.

DISCUSSION:

This protocol describes a method for producing MK capable of emitting proplatelets from blood-derived HP and to release platelets from the culture medium. HP are obtained from LRF, a by-product of the blood banks, used to remove contaminating leukocytes from cellular blood products and avoid adverse reactions. Although this method is relatively simple, a few points deserve special attention.

Deposition of the cell suspension on the density gradient medium (step 1.3.1) has to be performed gently to avoid mixture (red content). If this step is not carried out carefully, the protocol should stop at this point. Similarly, also in step 1.3.1, the brake has to be on the off mode to avoid mixing the fractions. If not, the HP selection must be suspended. As indicated in the protocol, section 1.4, the procedure can be interrupted following the PBMC collection. In this case, cells can be maintained under agitation overnight at 4 °C. Then, use a 40 µm cell strainer to remove the aggregates formed, which can impact on the subsequent CD34 selection. Of note, interrupting the procedure does not affect the yield and purity of $CD34^+$ cells. At the end of the CD34 selection, the purity must be greater than 75% to seed the cells since, in a previous study, differentiation and maturation of MK were poor below this purity[12] (**Figure 2**).

Special attention must be paid to the thawing of the $CD34^+$ cells, which must be carried out quickly to avoid affecting the viability of the cells. In addition, washing steps must be carried out carefully to leave no trace of serum. The cell seeding density must be respected as it has been rigorously chosen for optimal CD34 commitment to the MKP pathway and MK maturation (**Figure 3A**).

A cell phenotyping protocol is proposed to follow the differentiation and maturation of MK. This protocol is relatively basic, but it is important to have all the control tubes, both unlabeled and single labeling tubes, available for each day of analysis to ensure reliable settings on the cytometer. To ensure that the culture runs smoothly, it is important to collect information of proliferation along the procedure. At D7, the average proliferation is between 2 to 4 fold[13]. This proliferation varies little between experiments, since each LRF comprises cells from 8 donors. To smooth out the variations further, it is possible to combine cells obtained in parallel from 4 to 8 LRFs.

It is possible to look at the morphology of the cells by light microscopy but the cells should not be observed every day because they are sensitive to temperature variations. When removing the cells from the incubator, ensure to make slow movements to avoid breaking the proplatelets.

Concerning the platelet release, five times successive pipetting are required. Doing less does not ensure optimal platelet release and doing more is detrimental to their functionality[12]. The most important aspect in this step is to use precise and regular movements, to generate a regular flow required for the platelet release[14–16]. The method of five successive pipetting is, therefore, simple and easy to perform with satisfactory performance results based on the yields described in the literature. The number of released platelets can be determined as mentioned in the section 3.3 by using the strategy of flow cytometry analysis shown in **Figure 4B**. The quality of the released

platelets has been well documented in Do Sacramento et al. in terms of ultrastructure (morphology, size, granule content) and function (hemostasis), demonstrating that these cultured platelets are very similar to the native ones[13].

The protocol described here is particularly suitable for small-volume cultures but is not applicable to large-scale culture. It is therefore an optimal method for the study of platelet biogenesis in order to better understand the molecular and cellular mechanisms governing platelet production by adding small molecules, agonists, or antagonists, for example. In addition, and to further explore the mechanisms that regulate MK commitment, MK maturation, and platelet production, it is now possible to genetically manipulate the $CD34^+$ HP using a CRISPR-Cas9 genome editing method.

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DISCLOSURES:

The authors have nothing to disclose.

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16. Pallotta, I., Lovett, M., Kaplan, D. L., Balduini, A. Three-dimensional system for the in vitro study of megakaryocytes and functional platelet production using silk-based vascular tubes. *Tissue Engineering. Part C, Methods*. **17** (12), 1223–1232 (2011).

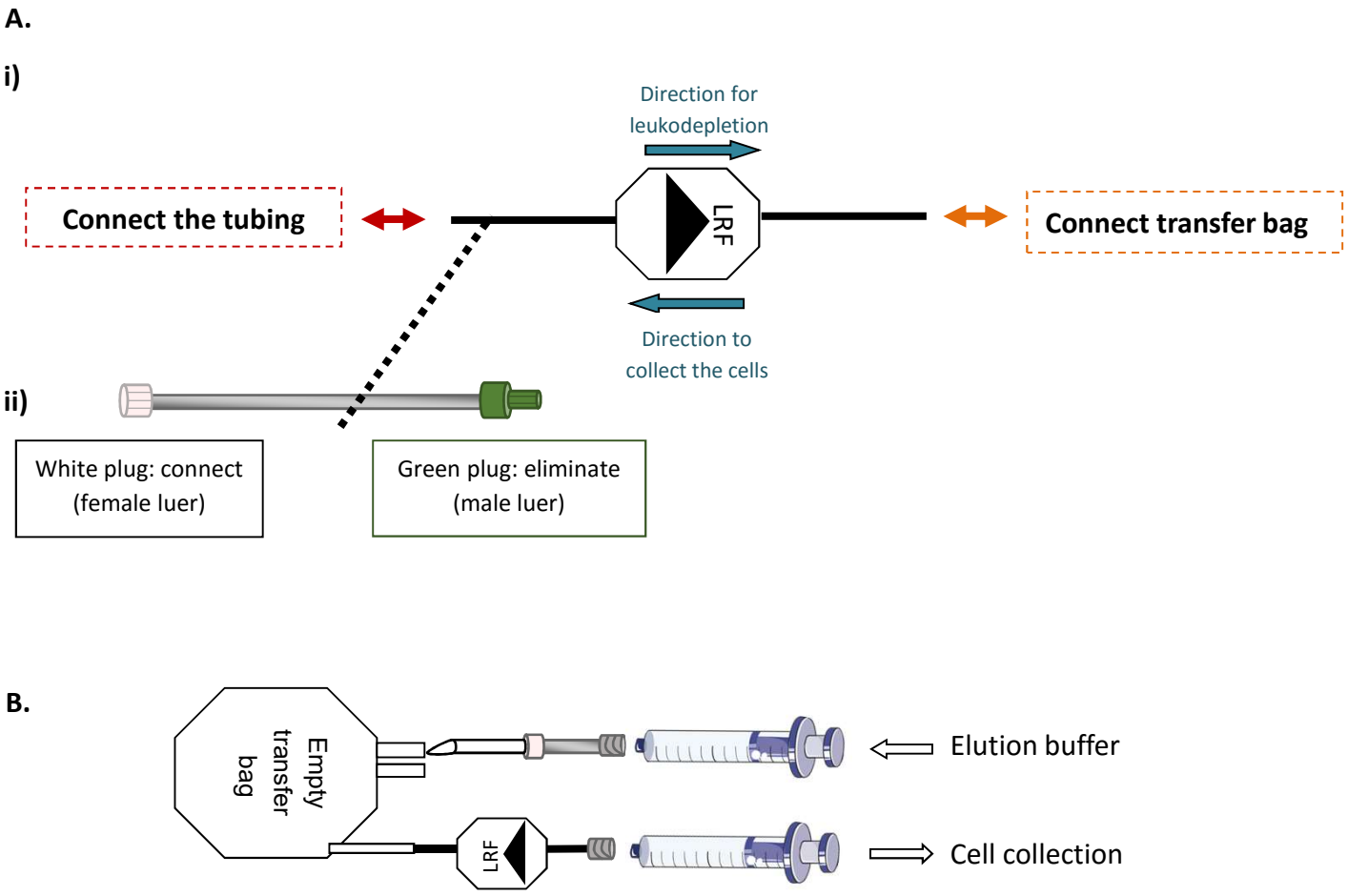
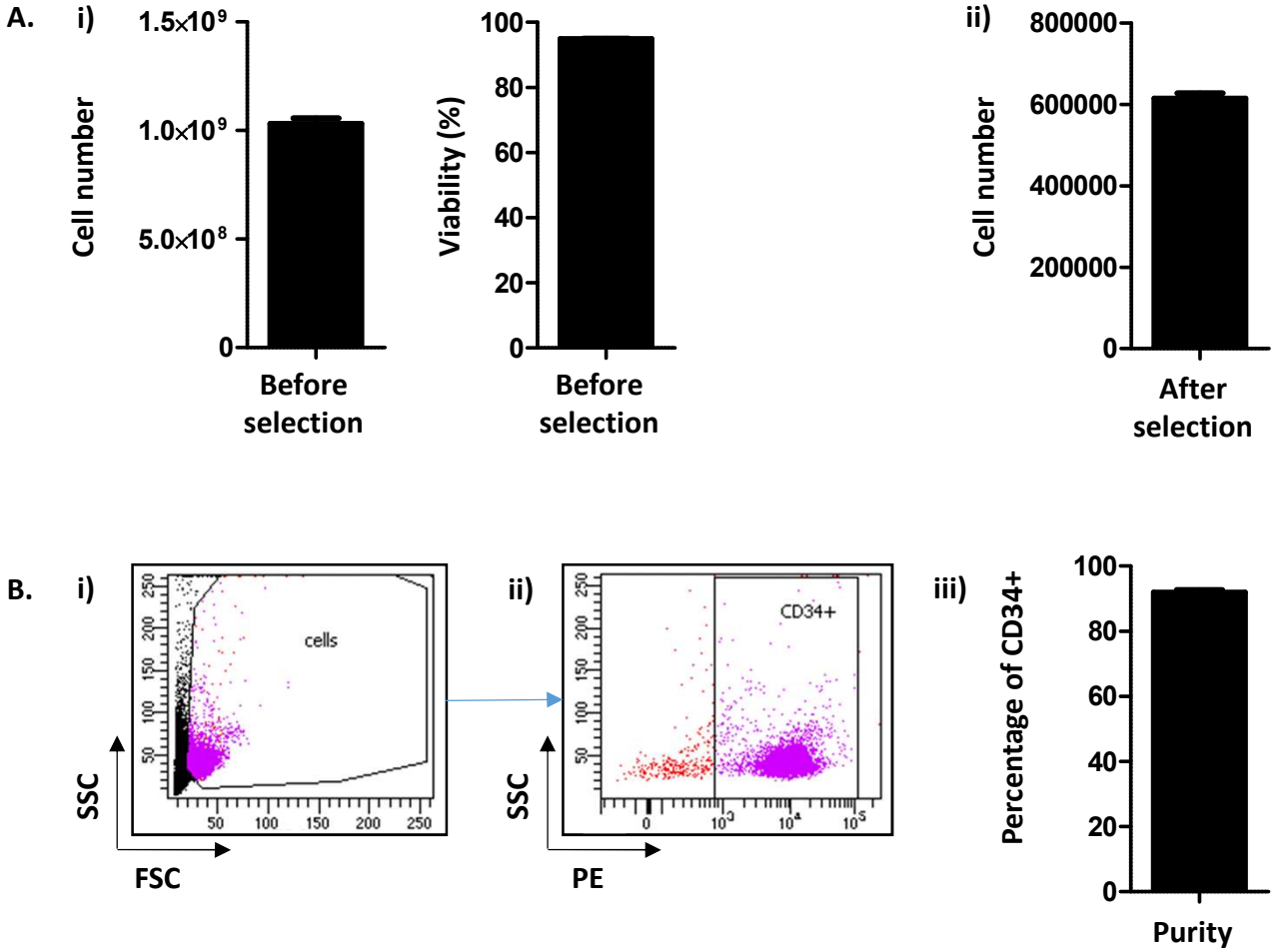


Figure 1. LRF backflushing modalities



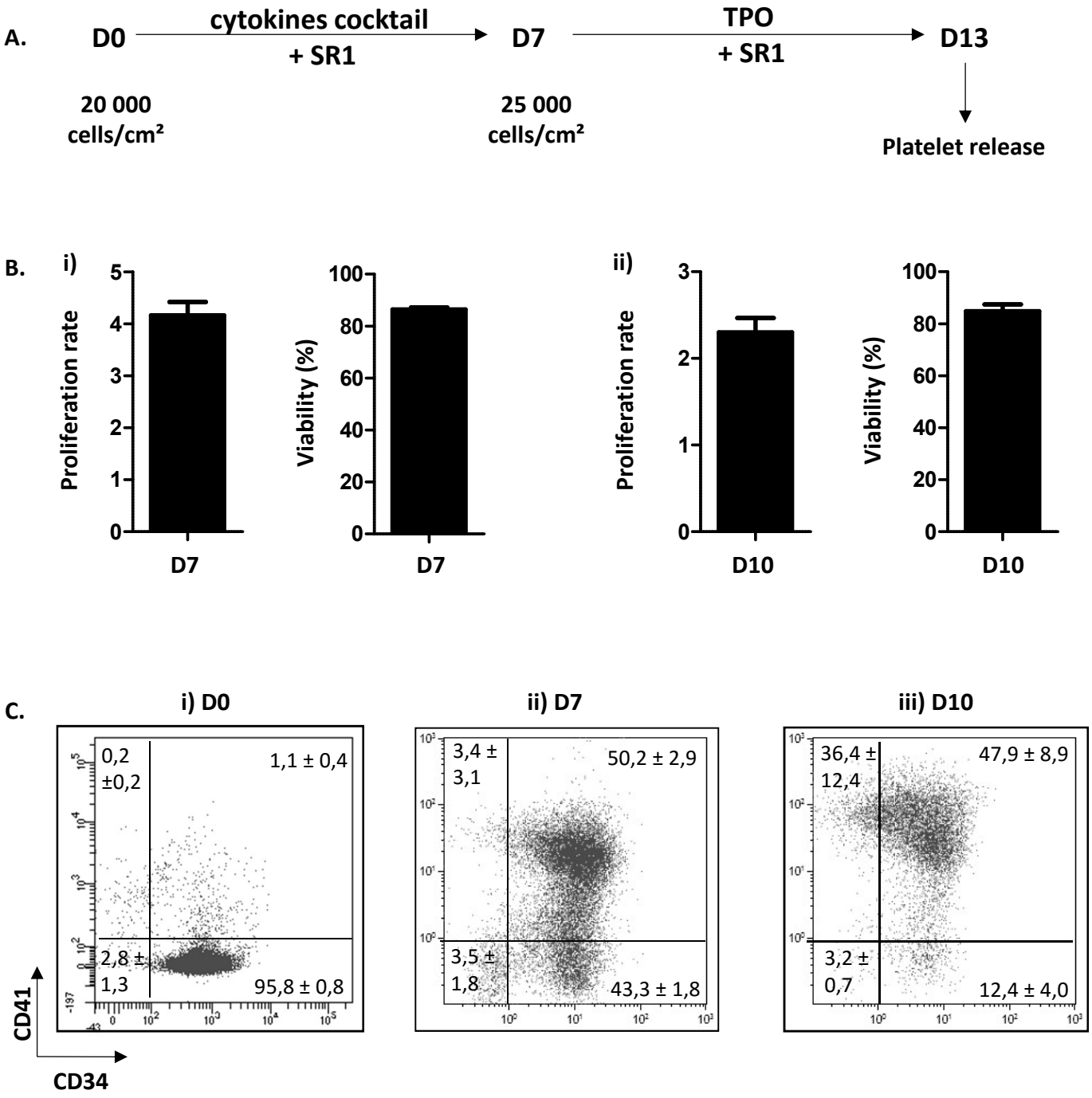
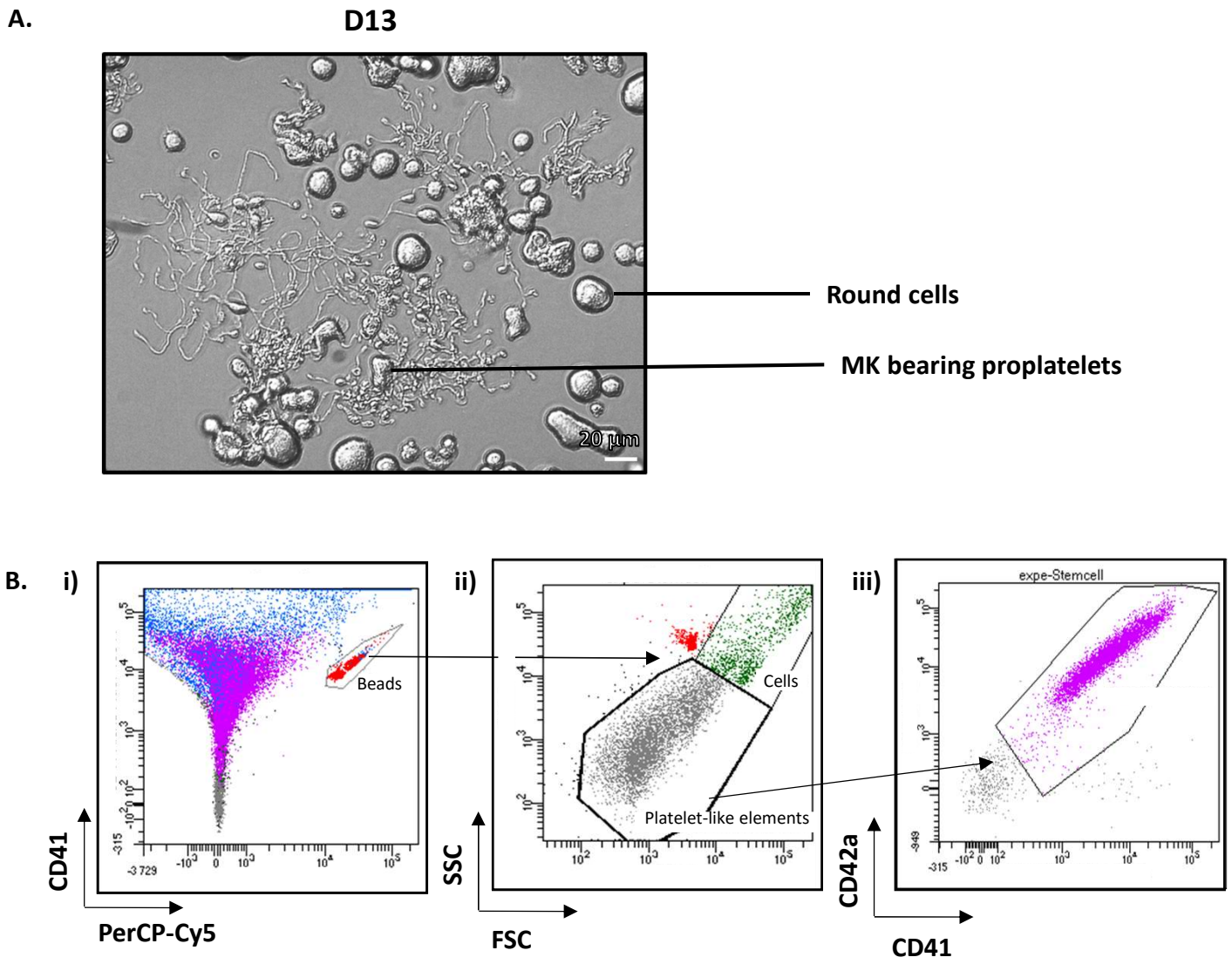


Figure 4

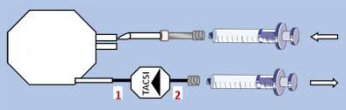


Isolation of CD34+

Preparation of solutions

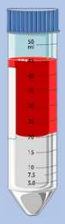
- Elution buffer: 85% PBS + 10% ACD + 5% FBS
- DGM preparation
- Dextran 2% at room temperature

Cell collection



- Transfer the eluate to a 50 mL tube
- Dilute with Dextran 2%

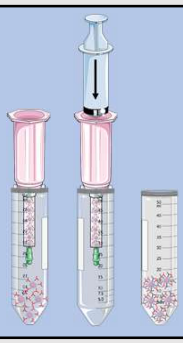
Buffy Coat



- Recover the supernatant and add it on top of the DGM
- Centrifuge at 400 g for 30 min with brake off
- Collect the PBMC fraction

- Washing steps (centrifugation 400 g for 10 min)

CD34+ selection



- Perform a cell count
- Incubate with the antibody for 30 min
- Washing step
- Pass the sample on the column (twice)

Freezing

- Perform a cell count and prepare:
  - 60% Stemspan + 40% FBS
  - 40% Stemspan + 40% FBS + 20 % DMSO
- Aliquot by 400 µL in cryotubes

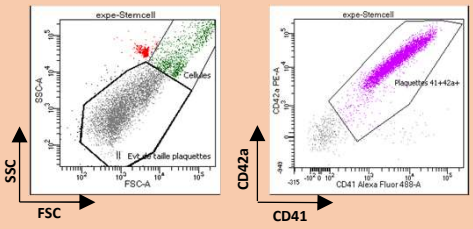
CD34⁺ Purity

- Incubate 100 µL of sample with 2 µL of hCD34-PE or IgG-PE antibody (for 30 min)
- Complete to 300 µL and perform flow cytometry analysis

Platelet Release

Release platelets

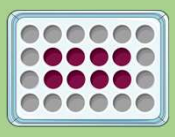
- At day 13
- 5 successive pipetting
- Perform cytometry tests



Cell Culture

Cell culture D0-D7

- Seed CD34+ medium / PSG / Human LDL / CC220 / SR1
- Incubate at 37°C for 7 days



Cell culture D7-13

- Seed cells in medium / TPO / Human LDL / SR1
- Incubate at 37°C for 6 days

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
7-AAD	Biolegend	558819	
ACD	EFS-Alsace	NA	
Anti-CD34-PE	Miltenyi biotec	130-081-002	
Anti-CD34-PECy7	eBioscience	25-0349-42	
Anti-CD41-Alexa Fluor 488	Biolegend	303724	
Anti-CD42a-PE	BD Bioscience	559919	
BD Trucount Tubes	BD Bioscience	340334	
CD34 MicroBead Kit UltraPure, human	Miltenyi biotec	130-100-453	
Centrifuge	Heraeus	Megafuge 1.OR	Or equivalent material
Compteur ADAM	DiagitalBio	NA	Or equivalent material
Cryotubes	Dutscher	55002	Or equivalent material
Dextran from leuconostoc spp	Sigma	31392-50g	Or equivalent material
DMSO Hybri-max	Sigma	D2650	
EDTA 0.5 M	Gibco	15575-039	
Eppendorf 1,5 mL	Dutscher	616201	Or equivalent material
Filtration unit Steriflip PVDF	Merck Millipore Ltd	SE1M179M6	
Flow Cytometer	BD Bioscience	Fortessa	
Human LDL	Stemcell technologies	#02698	
ILOMEDINE 0,1 mg/1 mL	Bayer	MA038EX	
Inserts	Fenwal	R4R1401	Or equivalent material
Laminar flow hood	Holten	NA	Archived product
LS Columms	Miltenyi Biotec	130-042-401	
Lymphoprep	Stemcell	7861	
Pen Strep Glutamine (100x)	Gibco	10378-016	
PBS (-)	Life Technologies	14190-169	Or equivalent material
Poches de transferts 600ml	Macopharma	VSE4001XA	
Pre-Separation Filters (30µm)	Miltenyi Biotec	130-041-407	
StemRegenin 1 (SR1)	Stemcell technologies	#72344	
StemSpan Expansion Supplement (100x)	Stemcell technologies	#02696	
StemSpan-SFEM	Stemcell technologies	#09650	
Stericup Durapore 0,22µm PVDF	Merck Millipore Ltd	SCGVU05RE	
SVF Hyclone	Thermos scientific	SH3007103	

Syringues 30 mL	Terumo	SS*30ESE1	Or equivalent material
Syringe filters Millex 0,22µM PVDF	Merck Millipore Ltd	SLGV033RB	
TPO	Stemcell technologies	#02822	
Tubes 50 mL	Sarstedt	62.548.004 PP	Or equivalent material
Tubes 15 mL	Sarstedt	62.554.001 PP	Or equivalent material
Tubulures	B Braun	4055137	Or equivalent material

Response to the reviewer

The changes appear in the text highlighted in green.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
This has been examined.

2. Please provide an institutional email address for each author.
The institutional email addresses have been provided.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.)
As requested by the editor, this has been modified throughout the manuscript.

4. Please define the abbreviations before use (ACD, PBMC, etc.)
As requested, the abbreviations have been previously defined.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: SVF Hyclone, TSCD, Terumo, Ficoll, Miltenyi Biotec, Stemspan, TrueCount, etc.
As recommended, all the commercial languages have been removed and products are mentioned in a table referenced in the material and methods section.

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.
As requested, a paragraph indicating the guidelines followed and our registration number with the competent authorities has been added (page 3, line 96-102).

7. Line 89/92/104/136/164: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2
The standard abbreviations have been used throughout the manuscript.

8. Line 109-111: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
As requested by the editor, the protocol was detailed in point 1.4 (page 4, line 136-144).

9. Line 119/195/207: Please elaborate the step for analyzing the purity of cells by Flow cytometry. If necessary, please cite a reference.
Analysis strategies for flow cytometry were described in the discussion section and in the legends of the figures concerned. For clarity a note has been added after the steps designating cytometry (Page 5 line 158, page 7 line 234 and page 8 line 248).

10. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the

video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and, therefore, will still be available to the reader.
As requested by the editor, the sections concerned by the video have been highlighted in yellow.

11. Figure 2A/3B: Please revise the Y-axis title from “Viabilite (%)” to “Viability (%)”.
This mistake has been corrected in Figure 2A and 3B.

12. Figure 5: Please remove the commercial names from the figure and replace them with generic names (e.g., Stemspan)
As requested by the editor, this has been modified in Figure 5.

Reviewer #1:

Manuscript Summary:
In the paper entitled "Leukodepletion filters-derived CD34+ cells: a cell source to study megakaryocyte differentiation and platelet formation "presented original article aiming Leukodepletion filters as a new source of CD34+ cells for research. The article is interesting and I think the figures were very helpful.,
comments:

1. What is the reason for using the empty bag connected to the filter during the back flushing step?
Two reasons justify the use of an empty bag: i) it is used as an intermediate storage bag to allow the elution buffer to run through the LRF, and ii) it allows the cells to be collected in a syringe after aspiration, facilitating their transfer into a 50 ml tube.

2. In my opinion, aspirating the buffer with a syringe through the filter causes pressure on the cells and changes them. If the filter is hanged, doesn't the cell suspension come out of the filter by gravity flow?
As pointed out by the reviewer, we could suspend the filter and not apply pressure, but this would be time consuming. Using a syringe allows us to recover the cells quickly and the viability analysis shows that there is no damage related to aspiration.

3. In this study, how much buffer was used to backflush the LRF?
As indicated in section 1.1 (page 3, line 105), the protocol is specified for 1 LRF corresponding to the preparation of 25 mL of elution buffer. For more clarity, a sentence has been added in point 1.2.1 (page 3, line 102-103) ”*Under the biosafety cabinet, inject the total volume of filtered and prepared elution buffer corresponding to the number of LRFs handled (xLRFX25 mL) into the empty bag”*

4. After the RBC precipitation, what volume of the cell suspension has been used for the MACS technique? Has it been affordable?

As requested by the reviewer, the detail of the technique has been added section 1.4 (page 4, line 136-144). The cost of the MACS kit is USD 1000.00.

5. What was the method of cell viability assessment?
A NANOENTEK Adam counter is used to give both the number of living cells and viability.

6. The Authors did not mention about the kind of filter and company name which is studied in this article and also it is better to mention with how much of the elution buffer the filter was washed.

At the request of the editor, commercial language has been removed and a generic term is to be used instead. We cannot therefore mention the model of the filter and the name of the company, but to answer the reviewer, the filter comes from the TERUMO TACSI system. The amount of elution buffer was discussed above and was specified in the manuscript in point 1.2.1.

7. I would recommend a rebuild introduction and underline the role of Leukoreduction filters as a new source of cells in the context of LRF's advantages

As suggested by the reviewer, the introduction has been reworked (page 2-3, lines 71-85).

8. Figure 1A/B: (treansfert bag) is not the correct word. Please correct.
This misspelling has been corrected in Figure 1B.

Reviewer #2:

Manuscript Summary:
The manuscript by Pongerard et al describes a method to culture megakaryocytes from CD34+ cells, enriched from the PBMC fraction of leuko-reduction filters, used to separate the blood components on each blood donation. The culture method facilitates as well as megakaryocyte maturation, platelet release from mature megakaryocytes. It is well structured, easy to follow, and useful for the scientific community. I only have some comments:

In the abstract, and introduction, it is mentioned that MKs can be grown and have been grown from various existing protocols, mainly from cord blood or bone marrow progenitors. It would be a more complete introduction when mentioning other existing protocols, for example (Salunkhe et al, Bio-Protocol 2015 DOI:10.21769/BioProtoc.1639).

We would like to thank the referee for his suggestion and the reference has been added to the manuscript and the introduction modified in page 2, line 71-75.

Major Concerns:
The authors suggest that platelet release can be favored by pipetting the MK mature fraction 5 times, up and down, with a P1000. The authors also suggest, not more, not less. However, the figure 4, does not show a comparison of increasing pipetting times, on the flow cytometry analysis of platelets, or count.

Could this be shown? Otherwise, why do the authors suggest that number? That would be very valuable to the manuscript.

This procedure has been published (Strassel C et al, Blood 2016) and patented (FR1557020). It offers the best balance between platelet yield per MK and platelet functionality. The corresponding

reference has been added in the discussion section page 12, line 388.

Minor Concerns:
There are some English style issues, although a grammar check-up is not required. One example is the use of "filtrated" instead of "filtered" in point 1.1.1.

As suggested, this has been modified, in point 1.1.1, page 3, line 106.

I would suggest to use Ficoll (with cap) in point 1.3.1, but I might be wrong.

As requested by the editor, commercial language was removed from the manuscript and a generic term was used instead. In section 1.1.3 (page 3, line 110), 1.3.1 (page 4, line 127) and 1.3.2 (page 4, line 129) Ficoll was replaced with « density gradient medium » (DGM).

In point 1.5.1., specify that an aliquot of the enriched fraction is used to verify the purity by FACS. Otherwise, some researchers might use the whole sample... It is not very clear.
We would like to thank the referee for his comment as the sentence was indeed not very clear and as suggested, this has been modified in point 1.5.1 (page 5, line 152) : *« Add to an aliquot of 100 µL of suspension obtained after the CD34+ selection… »*

In Figure 1B, transfer has an extra "t", Empty transfer bag (not transfert).
This spelling mistake has been modified in Figure 1B.

Reviewer #3:
Minor revision (more correct citation of precedent papers):
In this technical manuscript, the authors present a method to amplify and differentiate CD34+cells issued from leukodepletion filters (LDF) into megakaryocytes and platelets. Although this source of cells was already used for expansion of hematopoietic and progenitor cells in the context of development of hematopoietic graft (Haematologica 2018 103(10):1604-1615), as well as experimental ex vivo production of red blood cells there is no precise description of procedure for megakaryocytes production. Though, it was already showed that these CD34+ cells collected from filters can differentiate ex vivo in megakaryocytes (Transfusion, 2006 ; 46 :118-25), a fact that could be cited in this manuscript.

The procedure is very well described with enough details to enable a correct reproduction. I do not have any suggestion for improvement of the manuscript in addition to cite the historic publications.

We would like to thank the referee for his comments and as suggested references cited have been added to the manuscript page 2, lines 81-85.